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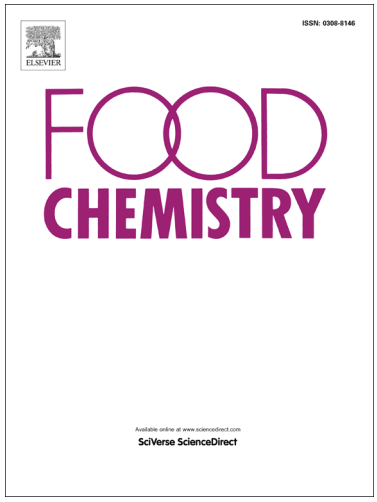


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# Structural analysis of the α-D-glucan produced by the sourdough isolate *Lactobacillus brevis* E25

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**Abstract**

Cereal-associated Lactic Acid Bacteria (LAB) are well known for homopolymeric exopolysaccharide (EPS) production. Herein, the structure of an EPS isolated from sourdough isolate *Lactobacillus brevis* E25 was determined. A modified BHI medium was used for production of EPS-E25 in order to eliminate potential contaminants. Analysis of sugar monomers in EPS revealed that glucose was the only sugar present. Structural characterisation of EPS by NMR and methylation analysis revealed that E25 produced a highly branched α-glucan with (α1→ 3) and (α1→6) glycosidic linkages, and was similar in structure to a previously reported EPS from *Lactobacillus reuteri* 180. The $^{1}H$ and $^{13}C$ NMR data were contrasted with newly recorded data for known polysaccharides (alternan, commercial dextran) which also contain α-(1,3,6)Glc branch points. It was found in both E25 EPS and alternan that NMR parameters could be used to distinguish glucose residues that had the same substitution pattern but occupied different positions in the structure.

**Keywords:** *Lactobacillus brevis,* exopolysaccharides, α-D-glucan, NMR analysis

## 1. Introduction

Lactic Acid Bacteria (LAB) have a long history in the production of fermented food products and have great importance today in the food industry for the bulk production of fermented foods such as dairy, meat and vegetable products and sourdoughs. Together with the production of organic acids by LAB during the early fermentation process, the generation of different metabolites such as bacteriocins, nutraceuticals and exopolysaccharides (EPS) is also of special interest due to their multifunctional roles in food products. Among these naturally produced components, EPS have unique functions in the development of physicochemical properties of fermented products such as sourdough. Several studies revealed the importance of *in situ* EPS production on the technological properties of sourdough and bread (Arendt, Ryan, & Dal Bello, 2007; Galle & Arendt, 2014). LAB strains can produce two types of EPS that can either encapsulate bacteria or be secreted into their surrounding environment. EPS can also be divided into two groups in terms of their structure: homopolysaccharides which are composed of only one type of sugar monomer and heteropolysaccharides, composed of two or more types of sugar monomer (Dertli et al., 2013). In general sourdough associated LAB produce homopolymeric type EPS comprising only glucose or fructose and forming different glucan and fructan structures (Galle et al., 2012). The production of glucan type EPS depends on the glucansucrase activity of LAB species which effects the hydrolysis of sucrose as the substrate and catalysis of transglycosylation reactions after the breakdown of sucrose resulting in the formation of polymeric glucans (Meng et al., 2016b; van Hijum, Kralj, Ozimek, Dijkhuizen, & van Geel-Schutten 2006). The production of different types of glucans with different linkages is related to differences in glucansucrase enzymes (Monchois, Willemot, & Monsan, 1999). So far several glucan types have been identified as being produced by different sourdough LAB strains (Bounaix et al., 2009). The main role of glucans as well as other EPSs in the sourdough environment is related to their influence on sourdough rheology and the textural characteristics of sourdough bread (Galle & Arendt, 2014).

Recently, we have isolated and identified novel LAB strains from traditional sourdoughs with the presence of *eps* genes related to both homopolymeric and heteropolymeric EPS production (Dertli, Mercan, Arıcı, Yılmaz, & Sağdıç, 2016). The aim of the present study was to characterise the exopolysaccharide (EPS) production characteristics of *Lactobacillus brevis* E25 selected from among the sourdough isolates. The sugar composition and structural characterisation of the EPS produced by E25 were determined by NMR and methylation analysis. Furthermore, the structure of E25 EPS was compared, via its NMR spectra, with structures of known polysaccharides (alternan and commercial dextran) containing glucose units with the same substitution patterns as E25. These findings may help us to understand the diversity of the EPS produced *in situ* during the sourdough fermentation process which may have an impact on the final functional roles of EPS in the sourdough environment.

## 2. Material and Methods

### *2.1. Bacterial Strains, Culture Conditions and Glucansucrase activity*

*Lactobacillus brevis* E25 as a sourdough isolate (Dertli et al., 2016) which has been deposited to Pamukkale University Food Engineering Culture collection (WDCM 1019) was grown under static conditions at 30 °C in MRS broth with 2% filter sterilized glucose as the carbon source for propagation purposes. In order to avoid potential contamination to EPS originating from yeast extract in MRS medium, E25 was grown in a modified BHI medium containing beef heart 5g/L, calf brains 12.5g/L, disodium hydrogen phosphate 2.5g/L, peptone 10g/L, sodium chloride 5g/L, glucose 10g/L, Sucrose 20g/L, Tween 20 1g/L, Sodium acetate 5g/L, Peptone casein 5g/L, Peptone meat 5g/L and Magnesium Sulfate 0.2g/L during EPS isolation process.

Additionally, the glucansucrase activity of *L. brevis* E25 was determined according to the previously described methodology (Bounaix et al., 2009).

### *2.2. Isolation of Exopolysaccharides*

*L. brevis* E25 was grown in modified BHI medium in 500 ml final volume, inoculated at 1% (v/v) with an overnight culture then incubated at 30°C for 2 d anaerobically. For the isolation of extracellular EPS, a previously described methodology was used (Dertli et al., 2013). Briefly, following the incubation 2 volumes of chilled ethanol was added to the culture supernatant obtained following the centrifugation of the bacterial culture and left at 4°C overnight to precipitate the EPS. The EPS pellet was then recovered by centrifugation at 10000 × g for 30 min at 4°C which was repeated three times with the use of less $H_2O$ at each time for the resuspension process. The final EPS pellet was resuspended in distilled water followed by dialysis for 72 h (12000–14000-Da visking dialysis membrane, Medicell International, UK) at 4°C, with two changes of $H_2O$ per day. The contents of the dialysis tubing were freeze-dried to provide EPS which was then used for structural analysis.

The alternan samples were kindly provided by the USDA Agricultural Research Service, Peoria IL 61604 USA and dextran from *Leuconostoc* spp. ($M_r$ 450,000-650,000, Sigma 31392) was from Sigma-Aldrich (UK).

### *2.3. Determination of Monosaccharide Composition by HPLC*

The monosaccharide composition of EPS produced by *L. brevis* E25 was analysed by HPLC (Shimadzu) using a CARBOsep CHO-682 Pb Column and RID-10A refractive index detector with a mobile phase of $H_2O$, flow rate 0.7 ml/min and column temperature of 25°C. Glucose, galactose, fructose, rhamnose were used as standard monosaccharides. The freeze-dried EPS was hydrolysed with 0.5 M $H_2SO_4$ at 95°C for 12 h followed by neutralisation with 4 M NaOH. The hydrolysates were filtered through a 0.45 µm pore size filter and the monosaccharide composition was determined by HPLC as described above.

### *2.4. NMR Spectroscopy*

NMR samples were prepared by dissolving 40 mg of each polysaccharide in 1 mL $D_2O$. Solutions (600 µL) were transferred to 5-mm NMR tubes for measurement. Spectra were measured at 600 MHz ($^1$H) and 150 MHz ($^{13}$C) using a Bruker Avance 600 NMR spectrometer equipped with a BBI probe or TCI cryoprobe. TOPSPIN 3.2 (Bruker) was used for all data acquisition and processing except the $^1$H spectral deconvolution which was carried out with MNova 10.0.2 (Mestrelab Research) software. Most spectra were recorded at high temperature (363$^0$K) using the BBI probe but some lower temperature spectra (320$^0$K or 338$^0$K) were recorded on the cryoprobe. Typical 90° pulses (BBI probe) were 9 µs ($^1$H) and 14 µs ($^{13}$C), and $^1$H spectra were acquired with presaturation of the residual HDO signal using standard Bruker methods and parameters (name of the pulse sequence is shown in italic type, followed by the number of scans for each experiment (NS)): $^1$H (*noesygppr1d*, NS = 64); $^{13}$C (*deptsp135*, NS = 25,000); COSY (*cosygpprqf*, NS = 8); TOCSY (*mlevphpr.2*, NS = 24, mixing time = 100 ms); HSQC (*hsqcetgpprsisp2.2*, NS = 48); HSQC-TOCSY (*hsqcdietgpsisp.2*, NS = 288, mixing time = 150 ms). Standard $^1$H spectra were recorded with the *noesygppr1d* sequence using an acquisition time of 2.62s, relaxation delay of 3s and water presaturation power level of 65dB. The parameters were slightly modified in spectra run at 320$^0$K to allow accurate integration: pulse sequence *zgpr*, relaxation delay 20s, presaturation power level reduced to 85dB.

Homonuclear 2D experiments were run with spectral widths of 12 ppm in both dimensions (or 3.5 ppm for higher resolution in TOCSY); heteronuclear experiments (HSQC, HSQC-TOCSY) were run with spectral widths of 12 ppm ($^1$H) × 70 ppm ($^{13}$C, range 50-120 ppm) acquired into 2048 (TD) × 1024 matrices and Fourier transformed with zero filling into 2048 × 2048 matrices. Spectra were referenced to the methyl signal of DSS ($\delta^1$H = 0 ppm, $\delta^{13}$C = 0 ppm) Note that on this scale, the chemical shifts of acetone are ($\delta^1$H = 2.208 ppm, $\delta^{13}$C = 32.69 ppm) and will be different from the values used by many authors in carbohydrate NMR.

*2.5. Methylation Analysis*

EPS linkage analysis was carried out via methylation by the method of Ciucanu and Kerek (1984), using finely ground sodium hydroxide that had been stored in a desiccator over phosphorus pentoxide. After acid hydrolysis with trifluoroacetic acid, methylated sugars were converted to peracetylated aldononitriles and analyzed by GC-MS on a Shimadzu QP2010 Ultra instrument with a Phenomenex Zebron HP1MS column (30meter length, 0.25mm diameter) (Seymour, Plattner, & Slodki 1975; Slodki, England, Plattner, & Dick. 1986).

## 3. Results

*3.1. Glucan producing sourdough isolate E25*

Cereal based LAB strains, especially those originating from sourdough are well known for their ability to produce homopolymeric glucan type EPSs. These characteristics are associated with the glucansucrase activity of LAB and several sourdough LAB strains have been shown to have this type of activity leading to glucan type EPS production (Bounaix et al., 2009). Our previous report also revealed the potential of sourdough LAB isolates to produce both homopolymeric and heteropolymeric species as all of our isolates harboured *eps* genes related to both types of EPS production (Dertli, Mercan, Arıcı, Yılmaz, & Sağdıç, 2016). In this study we focused on the structural characterisation of EPS produced by *L. brevis* E25 selected from

the sourdough isolates. In general, glucansucrase activity occurs in the culture supernatant and glucan type EPSs are produced within the culture supernatants of LAB strains. One of the main drawbacks of the EPS isolation process associated with the cultivation of LAB strains in MRS broth is that the required EPS may be contaminated with yeast cell-wall glucan present in the medium. In order to avoid this problem, we have developed a modified-BHI medium that does not contain any potential contaminant related to the yeast extract. *L. brevis* E25 grew well in this medium compared to MRS (data not shown) and EPS from *L. brevis* E25 was isolated from the culture supernatant of this medium.

The HPLC analysis of the isolated EPS revealed that EPS E25 contained glucose as the only sugar monomer (data not shown). This result confirmed that the strain produced only a glucan type EPS potentially due to its glucansucrase activity as previously discussed (Bounaix et al., 2009; Kralj et al., 2003). The glucansucrase activity of *L. brevis* E25 observed in cell associated and soluble fractions was found to be 1.631 ± 0.28 and 0.208 ± 0.05 U. $ml^{-1}$, respectively. Similar to *L. brevis* E25, other LAB strains were also shown to have higher levels of cell associated glucansucrase activities than their soluble fractions (Bounaix et al., 2009). Previously, *Lactobacillus brevis* G-77 was also shown to produce glucan type EPS (Mårtensson, Dueñas-Chasco, Irastorza, Öste, & Holst 2003).

Having established that a glucan was produced by *L. brevis* E25, the structure of the E25 EPS was determined by NMR and methylation analysis. Interpretation of the data was aided by comparison with three related glucans: alternan from *Leuconostoc citreum* NRRL B-1355 (Misaki et al., 1980; Seymour et al., 1979a); a commercial dextran; and the well characterised EPS180 from *L. reuteri* 180 (van Leeuwen et al., 2008).

*3.2. Methylation Analysis*

Methylation analysis (Table 1) showed that the E25 polysaccharide was composed of terminal, (1,3)-disubstituted, (1,6)-disubstituted and (1,3,6)-trisubstituted glucose residues. This combination of residues was the same as that found in the B1355S and B1501S glucans isolated from *Leuconostoc citreum* strains NRRL B-1355 and NRRL B-1501 (Jeanes et al., 1954; Seymour et al., 1979b; Seymour, Slodki, Plattner, & Jeanes., 1977). (The same strains produce in addition the polysaccharides B1355L and B1501L which are dextrans containing over 90% (1,6)-disubstituted glucose residues (Seymour, Chen, & Bishop, 1979)). Methylation analysis, NMR and degradation studies showed that B1355S and B1501S contained α-linked glucose units with the two types of disubstituted residue linked in an alternating sequence, →6)-α-Glc-(1→3)-α-Glc-(1→, and these polysaccharides were given the name alternans (Côté & Robyt, 1982) to distinguish them from dextrans. Methylation analysis results for E25 are compared in Table 1 with those for the alternans (re-determined for this work so that exactly the same procedure was used for the three samples) and with literature data for the polysaccharide EPS180 (van Leeuwen et al., 2008). The ratio of (1,6)-:(1,3)-Glc residues in E25 is ~2.9:1 whereas for the two alternans it is 1.1:1 or 1.4:1. The composition of E25 appears to be much closer to that of EPS180 (van Leeuwen et al., 2008) than to the alternans (Côté, 2002). A difficulty with the methylation results for E25 is the large difference in values found between t-Glc and (1,3,6)-Glc. It would be expected that the

number of terminal residues would equal the number of branch points and whilst it can be seen from the alternan results that some underestimation of the branch point residues is not atypical, the discrepancy is much greater for E25. The reason for this is not clear but it is safe to conclude that there is a much greater excess of (1,6)-Glc over (1,3)-Glc residues and (based on the t-Glc percentage) a higher degree of branching in E25 compared with either B1355S or B1501S.

*3.3. NMR Spectroscopy*

*3.3.1 $^{1}$H NMR*

The NMR spectrum of E25 was also compared with that of authentic alternan, B1355S, and with a commercial dextran. The dextran was included as a simple example of a polysaccharide containing (1,3,6)-α-Glc branch points as assignment of the corresponding units was not easy in the other two polysaccharides. Spectra were recorded at three different temperatures ($320^{0}$K, $338^{0}$K, $363^{0}$K). The general line-narrowing effect of increased temperature was only modest but the variation of certain chemical shifts with temperature gave additional resolution in parts of both the $^{1}$H and $^{13}$C spectra. The $^{1}$H NMR spectra of E25 and B1355S at $320^{0}$K and $363^{0}$K are shown in Fig. 1. The spectra show features in common with spectra reported for EPS180 (van Leeuwen et al., 2008) and polysaccharides from three strains of *L. citreum* found in sourdough (Bounaix et al., 2009). The glucose residues present are distinguishable by NMR according to their substitution patterns and linkages.

In common with previous work (van Leeuwen et al., 2008) the residues are coded as follows: **A** (1,3)Glc→6; **B** (1,6)Glc→3; **C** (1,6)Glc→6; **D** t-Glc→6; **E** (1,3,6)Glc→6; **F** t-Glc→3. The notation (1,3)Glc→6 etc. is an abbreviation for –(1→3)-α-D-Glc*p*-(1→6)-. t-Glc→3 indicates a terminal non-reducing end residue i.e. α-D-Glc*p*-(1→3)-. The context will make it clear whether **C**1 refers to H-1 or C-1 of unit **C** etc. The H-1 signals at 5.3 ppm and 5.0 ppm are readily identified as arising from (1→3) and (1→6) linked α-Glc units respectively: integration of the $320^{0}$K spectra gives the ratio of (1→3): (1→6) linked units as 1: 2.0 and 1: 1.48 for E25 and B1355S respectively. The value for EPS180 was given (van Leeuwen et al., 2008) as 1: 2.2. Two other groups of signals are specific for particular hydrogen atoms and were also seen in the spectrum of EPS180 (van Leeuwen et al., 2008). The first of these is the H-5 of unit **B** which has the unusually high chemical shift of 4.15-4.20 ppm (this doublet is seen most clearly in the $320^{0}$K spectra of Fig. 1, at $363^{0}$K its intensity is diminished by presaturation of the neighbouring water peak. The **B**5 chemical shift is temperature dependent). The unusual chemical shift is attributable to hydrogen bonding between O-5 of the (1,6)-α-Glc→3 unit and H(O)-2 of the neighbouring 3-linked Glc (van Leeuwen et al., 2008). The second group of signals is a set of overlapping triplets ($\delta_H$ 3.43-3.45 ppm) attributable to H-4 of the terminal α-Glc groups. In EPS180 only t-Glc→6 (**D**) units were detected (van Leeuwen et al., 2008) but we also found t-Glc→3 (**F**) units in both E25 and B1355S with the chemical shift of **F**4 slightly higher than that of **D**4. These 'reporter' signals were valuable starting points for the full assignment of the $^{1}$H and $^{13}$C spectra of the polysaccharides. It is also seen in Fig. 1 (b, d) that the peak at 5 ppm is resolved into two

signals at the higher temperature and that the two have different relative intensities in E25 and B1355S.

*3.3.2 $^{13}C$ NMR*

The $^{13}C$ NMR spectra (*dept135* sequence) of E25, B1355S and dextran at $363^0K$ are shown in Fig. 2. The spectra of B1355S and several dextrans were reported previously (Seymour et al., 1979a) but because they were recorded at lower field strength they did not show the level of detail revealed here. However, based on the previous work a number of the signals falling in the different spectral regions shown can be identified. In the anomeric region the signals can be divided into those originating from Glc(1→3) units (**B1, F1**, ~102 ppm) and from Glc(1→6) units (**A1, C1, D1, E1**, ~100.8 ppm). The signals at ~84.5 ppm are from C-3 of 3-linked Glc (**A3, E3**) with C-3 of the remaining units (**B3, C3, D3, F3**) at ~76 ppm. The 72-75 ppm region requires 2D NMR for assignment but the inverted C-6 signals are readily divided into those from C-6 linked Glc (**B6, C6, E6**, ~68.7 ppm) and the remaining Glc units (**A6, D6, F6**, ~63.4 ppm). The assignment of the signals from the predominant (1,6)-α-Glc→6 units in dextran is well known and these are labelled **C1-C6** in Fig. 2c.

*3.3.3 B1355S – 2D NMR and assignments*

The detailed assignment (Table 2) of $^1H$ and $^{13}C$ chemical shifts in E25, B1355S and the minor units of dextran was obtained mainly from the $^1H$-$^{13}C$ 2D experiments, *hsqc* and *hsqc-tocsy*, with some help from *cosy* and *tocsy* experiments for the $^1H$ assignment. The *hsqc-tocsy* spectrum of B1355S is shown in Fig. S1 with the $^{13}C$ axis divided into segments similar to those shown in Fig. 2. The absence of signals in the 84 ppm region (Fig. S1b) on the vertical through $\delta_H = 5.3$ ppm indicates that there are no (1,3)-α-Glc→3 or (1,3,6)-α-Glc→3 units present in B1355S as both units would have a $^{13}C$ signal in the region. The same conclusion was drawn for E25. Based initially on identification of the reporter signals mentioned above it was possible to obtain all the $^1H$ chemical shifts of units **B** and **F** from the horizontal traces at $\delta_C$ ~ 102 ppm (Fig. S1a). The $^1H$ shifts of units **C** and **D** were obtained from the horizontal traces at $\delta_C$ ~ 76 ppm (Fig. S1c). All four C-3 signals, associated with the four units that are not 3-linked, were resolved in this group. The two 3-linked units, **A** and **E**, have their C-3 signals at $\delta_C$ ~ 84.5 ppm. The $^1H$ shifts of the **A** unit may be obtained from the horizontal trace with the highest contour levels (labelled **A** in Fig. S1b) or from the trace in the anomeric region ($\delta_C$ ~ 101 ppm). At a slightly higher shift ($\delta_C = 84.72$ ppm) there is a trace which includes a $^1H$ signal at 3.95 ppm. This is the expected chemical shift for one of the H-6 protons of a 6-linked Glc (the H-2-H-6 chemical shifts of **A** all fall in the range 3.65-3.85 ppm). Therefore, the trace must come from a unit which is both 3- and 6-linked, i.e. it must be the (1,3,6)-α-Glc unit, **E**, with $\delta_C$(**E**3) = 84.72 ppm. Note that no clear signal is obtained at this chemical shift in the 1D $^{13}C$ spectrum. Evidently the mobility of the branching unit is restricted enough to severely broaden the **E**3 signal; the **A**3 signal is also broadened relative to other signals but not to the same degree. After suitable horizontal traces have been identified for each unit it is possible to label the various vertical traces at $\delta_H$ ~5.3 and ~5.0 ppm (see Fig. S1). In particular, it shows that the splitting of the group at $\delta_H$ ~5.0 ppm, which

is resolved at $363^0$K but not at $338^0$K, results because **A**1 and **E**1 have slightly higher chemical shifts than **C**1 and **D**1.

Sequential assignments (H-2, H-3…) of the individual units are made from the *cosy* spectrum (not shown) starting from either the cross peaks involving anomeric protons or those involving the reporter signals mentioned above such as **B**5, **D**4 and **F**4. $^{13}$C chemical shifts of each unit are read off from the *hsqc-tocsy* spectrum (Fig. S1) using either the vertical traces in the $^1$H anomeric region or other convenient traces, again involving the reporter signals (e.g. the **B**5 vertical line at 4.13 ppm for unit **B**). Then the $^{13}$C assignments (C-2, C-3…) are completed using the $^1$H assignments together with the *hsqc* spectrum. Fig. S2 shows the labelled $^1$H-$^{13}$C cross peaks for the most crowded region of the *hsqc* spectrum. The results are collated in Table 2 and are generally in accord with reference results (Jansson, Kenne, & Schweda, 1988) for oligosaccharides: for example δ(H-2) is downfield for the 3-linked Glc residues ($\delta_H \geq 3.65$ ppm for **A**2 and **E**2) compared with the other Glc units; $\delta_H$ is considerably downfield for **F**5 (t-Glc→3 unit, δ = 3.98) compared with **D**5 (t-Glc→6, δ = 3.71, see Fig. S2). One additional feature of the *hsqc-tocsy* spectrum, not mentioned so far, is that at least three horizontal traces (with similar sets of $^1$H shifts) are seen for certain $^{13}$C signals such as **B**1 (Fig. S1a) and **A**3 (Fig. S1b). The possible reason for this becomes clearer when the spectra of B1355S are considered in relation to those of E25.

*3.3.4 E25 – 2D NMR and assignments*

The assignment of the $^1$H and $^{13}$C chemical shifts of E25 followed the same procedure as that described for B1355S. The essential similarities between E25 and B1355S are reflected both in the *hsqc-tocsy* (Fig. S3) and *hsqc* (Fig. S4) spectra of E25 and the corresponding figures for B1355S as well as in the values recorded in Table 2. The main differences lie firstly in the increased population of residues of type **C** [(1,6)-α-Glc→6] in E25 compared with B1355S, and secondly in the greater degree of branching in E25. Both these features are illustrated by the assigned $^{13}$C spectra of Fig.2: the first by the increased intensity of the signals labelled **C**1-**C**6 in E25 and the second by the increased ratio of (**D**6 + **F**6): **A**6 in E25. In the latter case (**D**6 + **F**6) represents the total of terminal residues which is equal to the number of branch points whereas **A**6 represents the number of 'internal' residues in a branch (the ratio is also affected by the diminished proportion of **A** residues in E25, as shown in *3.3.7*). Both differences agree with the main conclusions drawn from methylation analysis.

*3.3.5 Dextran – 2D NMR and assignments*

The spectra of the dextran sample were also assigned in the same way. It can be seen from Fig.2 (and Table 2) that there is good agreement between the chemical shifts found for dextran, E25 and B1355S, for both the major signals of dextran (**C**1-**C**6) and its minor signals. The assignments of the **E** unit signals in the *hsqc* spectrum of dextran (Fig. S5) lent support to the assignments of the corresponding **E** signals found at similar locations in the *hsqc* spectra of the other two polysaccharides. The NMR results also showed that at least two types of (1,3,6)-α-Glc branching unit could be distinguished in dextran thanks to the separate signals observed for C-3 at $\delta_C \sim 84$ ppm. These signals are labelled **E**3 (itself two signals) and

**E**3´ in Fig. 2c. **E**3´ has the same integral value as the anomeric signal **F**1 whilst **E**3 has the same integral as **B**1. Taking into account its other chemical shifts the unit **F** is t-α-Glc→3 and therefore the **E´** unit must be a (1,3,6)-α-Glc substituted at O-3 by a single α-Glc residue. Chemical degradation studies of the dextran NRRL B-512 showed that 40% of side chains consisted of just a single Glc residue (Larm, Lindberg, & Svensson, 1971). The unit labelled **E**, on the other hand, must be a branching unit substituted by a chain with more than one Glc (the degradation studies indicated that branches of just two glucose units were present as well as longer chains) in which case the first unit of the side chain will be of the **B** type [(1,3)-α-Glc→6] with the anomeric signal **B**1 (Fig. 2c).

*3.3.6 Nearest neighbour effects. E25 is a single polymer, not a mixture.*

It was mentioned above that multiple $^{13}$C signals were detected for **B**1 and **A**3 in B1355S (Fig S1a, S1b). This feature is more pronounced in E25 (Fig S4a, S4b) and can be seen directly in Fig 2a where splittings of the **B**1, **A**3 and **B**5 resonances are evident. The *hsqc-tocsy* anomeric regions (Figs S3a, S1a) show that there are two **A**1 $^{13}$C resonances at the same **A**1 $^{1}$H chemical shift and a comparison of intensities (e.g. of **A**1 and **B**1 in E25 versus their counterparts in B1355S, Fig 2a, 2b) makes it clear that the **A**1 signal is split, although the second **A**1 signal is hidden by **C**1 in E25. The 'new' signals in E25 are labelled **A´**, **B´** in the $^{13}$C projection area of Fig S3 whilst the signals that have the same chemical shifts in E25 as in B1355S are labelled **A**, **B**. In fact, the **A´** and **B´** signals are present in B1355S but are weak whereas in E25 the **A´** and **B´** sets are stronger than **A** and **B** (see the relevant contour levels in the *hsqc-tocsy* spectra or the intensities of **B**1 and **B**5 in Fig 2). The presence of multiple signals from sugar units with the same substitution pattern implies that the units have different locations (e.g. different nearest neighbours). Based on model structures of alternan (Misaki et al., 1980) and EPS180 (van Leeuwen et al., 2008) it seems reasonable to distinguish between 'internal' units (i.e. **A** in the sequence **-BAB-** or **B** in the sequence **-ABA-**) and those adjacent to branching residues (i.e. **A** linked via O-6 or **B** linked via O-3 to an **E** unit). Then the units labelled **A´**, **B´** correspond to those adjacent to a branching residue while **A, B** are the internal units. In B1355S the main signals are from the internal units whereas in the more highly branched E25 the **A´**, **B´** signals are slightly stronger than those from the internal units. The situation is rather more complex however since there are three $^{13}$C traces for both **B1** and **A3** in the *hsqc-tocsy* spectra, including an **A3** outlier at $\delta_C$ ~ 83.6 ppm. This complexity may concern the **C** residues and how they are accommodated. In one alternan model (Misaki et al., 1980) **C** units are inserted prior to some bridging units to give sequences of the type –**ACE-** (in addition to –**BAE-**) and the same is found in the EPS180 model (van Leeuwen et al., 2008) which also includes –**CBE-** (in addition to –**ABE-**) and –**CCC-** sequences of variable length. Sequences including the **C** unit will provide new nearest neighbour combinations for the **A** and **B** residues which may add to the number of $^{13}$C signals present for these residues, particularly for the linkage carbons. The NMR data on the intact polysaccharides does not determine the frequency of such sequences but an estimate can be made (without recourse to the methylation analysis results) of the relative proportions of the individual units (*3.3.7*).

It was mentioned in *3.2* that the strains NRRL B-1355 and NRRL B-1501 produce dextran as well as alternan. The two polysaccharides have to be purified by differential precipitation from ethanol/ water mixtures. It is important to question therefore whether E25 could be a mixture of dextran and alternan rather than a single polymer. A comparison of the splitting patterns and linewidths of signals (**B**1, **A**3, **B**5) discussed in this section for the three polysaccharides shown in Fig 2 confirms that E25 is an independent polymer with features that differentiate it from alternan and dextran. Its $^{13}$C spectrum could not be reconstructed as a combination, in any proportion, of the spectra of the other two polysaccharides.

*3.3.7 Percentages of individual units in E25 and B1355S*

The four labelled peaks (or peak groups) in the 320$^0$K $^1$H spectra of E25 and B1355S were integrated (Fig. 1a, 1c) relative, in each case, to an arbitrary value of 1.0 assigned to the peak at $\delta_H$ ~5.3 ppm. The NMR measurement parameters were set to make these integrals as reliable as possible. Integral ratios were also obtained by deconvolution from the spectra taken at 363$^0$K (to resolve the **A**1+**E**1 and **C**1+**D**1 signals, Fig 1b, 1d) and 363$^0$K or 338$^0$K (to better resolve **D**4 and **F**4 signals). Results are reported in Table 3A. It is then possible to derive a value (in integration units) for each type of Glc residue, **A**-**F**. Using a deconvolution ratio, the contributions of **D** and **F** to the (**D**4+**F**4) integral can be obtained, then since **F** is known the contribution of **B** to the (**B**1+**F**1) integral is calculated. If the integral value of **E** (branching unit) is then made equal to the sum of the terminal units (**D** + **F**) the integral values appropriate to **A** and **C** may be calculated from the deconvoluted (**A**1+**E**1) and (**C**1+**D**1) integrals. The **B**5 integral was not used in the calculation but served to check the consistency of the results. The derived integral values are reported in Table 3B together with the percentages of each unit calculated from these values. The results are subject to some uncertainty because of the need to deconvolute signals with non-ideal lineshapes but if the percentages are summed for the two (1,6)-linked and the two terminal residues a comparison can be made with the completely independent methylation analysis results in Table 1. For E25 the values for (1,3)-Glc and (1,6)-Glc are in good agreement whilst the NMR values for (1,3,6)-Glc and t-Glc, which were constrained to be equal, fall between the very different values obtained from methylation analysis. For B1355S the NMR finds (1,3)-Glc somewhat lower and (1,6)-Glc somewhat higher than the methylation analysis although there is good agreement between the methods for (1,3,6)- Glc and t-Glc. Based on Table 3B there is a marked decrease in the proportion of **A** residues in E25 compared with B1355S, together with a smaller decrease of **B**, an increase in branching/ terminal residues and a notable increase in the proportion of **C**. The 'average repeating unit' of E25 (meaning the interval between branching unit and terminal residue or between branching units) will then consist of approximately 2 **B** units, 1 **A** unit, and 1-2 **C** units whereas in B1355S it will be 3 **B** units, 2-3 **A** units and 1 **C** unit. This explains why the $^{13}$C NMR signals assigned to the 'internal' **A** and **B** units in B1355S are diminished in E25. Clearly the structure of E25 departs considerably from that of the classical alternans, B1355S and B1501S both through the shortening of the 'average repeating unit' and the substitution of **C** units (mostly for **A** units) that disrupts the characteristic **AB** repeating sequence.

## 4. Discussion

It is known that the glucansucrases from *Leuconostoc mesenteroides* NRRL B-512 (dextransucrase, DSRS), *Leuconostoc citreum* NRRL B-1355 (alternansucrase, ASR) and *L. reuteri* 180 (GTF180) have many common features including their domain organisation, a series of conserved sequence motifs in their catalytic domains, and a common mechanism of action (Meng et al., 2016b; Moulis et al., 2006). In view of the similarity of its polysaccharide product to EPS180, it seems certain that the hitherto uncharacterised glucansucrase from *L. brevis* E25 belongs to the same category. For this reason, detailed studies of GTF180, its mutants and the polysaccharides they make are of interest (Meng et al., 2016b). The synthesis of α-glucan from sucrose is accomplished by a two-step reaction in which the linkage between Glc and Fru is cleaved and the Glc unit forms a covalent intermediate via its anomeric carbon with the enzyme; in the second step the enzyme-bound Glc anomeric carbon is attacked by one of the free hydroxyl groups from the non-reducing end of the growing α-glucan chain, releasing the Glc from the enzyme and adding it via an α-linkage to the chain. The catalytic function is carried out by the same three amino acid residues in all LAB glucansucrases: an Asp (NU, nucleophile) that makes the glucose-enzyme linkage, a Glu (A/B, acid/ base residue) that provides a proton (step 1) and then accepts a proton (step 2), and a second Asp (TS) that stabilises the transition state (Meng et al., 2016b; Moulis et al., 2006). In this reaction the Glc unit is termed the *donor* and the growing glucan the *acceptor*.

Glucansucrases from different bacterial strains utilise differing proportions of sucrose for α-glucan synthesis and hydrolysis. They also produce a wide variety of α-glucans with different physical properties; linkage types (polysaccharides with different proportions of α1→6, α1→3, α1→4, α1→2 linkages are known); degrees of branching; and polysaccharide to oligosaccharide product ratios. The crystal structure of GTF180-ΔN (Vujičić-Žagar et al., 2010) together with studies in which the residues making up the carbohydrate binding sites have been mutated (summarised in Meng et al., 2016b) have shown that the identities of as many as 15 binding site residues can play a part in determining the orientation of the acceptor non-reducing Glc ring and its neighbour(s) with consequences for the linkage specificity and branching density.

With so many factors able to affect profoundly the structures of the resultant EPSs it is reasonable to ask whether the E25 EPS and EPS180 are in fact identical. We do not yet have sequence data available for the E25 glucansucrase and although the methylation analysis results for the two polysaccharides are similar they are subject to experimental error. Detailed comparison of the $^{13}$C NMR spectra of the two polysaccharides would be one way to answer the question but the $^{13}$C spectrum of EPS180 does not seem to have been published. Van Leeuwen et al. (2008) described the EPS180 structure as 'built-up from different lengths of isomalto-oligosaccharides, interconnected by single (α1→3) bridges'. They provided a composite model structure based on $^{1}$H NMR, methylation analysis and partial acid hydrolysis results. The fragments characterised after acid hydrolysis included isomalto-oligosaccharides up to isomaltoheptaose and such relatively long chains of consecutive (α1→6) linked Glc units were incorporated in the model including, at intervals, (1,3,6)Glc→6 branch points

where the (α1→3) link to the branch unit is presumed to be cleaved during hydrolysis. We have worked exclusively with the intact polysaccharide and do not have comparable acid hydrolysis results but have attempted to provide a model structure of E25 EPS (Fig. 3) which retains this feature of the van Leeuwen et al. (2008) model and also displays the proposed location of the **A´** and **B´** units discussed in *3.3.6*. One difference between E25 (or B1355S) and EPS180 is that the first two polysaccharides have both t→3 and t→6 Glc units whereas EPS180 has only t→6 Glc. It is interesting to note that many of the mutations to residues at the +2 binding site (Meng et al., 2016a; van Leeuwen et al., 2009) in GTF-ΔN introduce t→3 Glc units into the modified EPSs that are produced although most of these EPSs also include t→4 Glc units which are definitely not present in E25 or B1355S.

In conclusion, we have provided a detailed characterisation of an exopolysaccharide produced from sucrose by *Lactobacillus brevis* E25 from sourdough. Similar products were present among those identified previously in a screening study of polysaccharides from sourdough lactic acid bacteria and probably play a functional role. At the time their structures were tentatively likened to that of alternan (Bounaix et al., 2009). We have shown that the structure of E25 EPS more closely resembles that of EPS180 from *Lactobacillus reuteri* 180. Understanding of how glucansucrases extend glucan chains and incorporate different linkage specificities has advanced greatly and it is becoming clearer, at least in oligosaccharide models, how the branch points might be introduced. However, the factors that control branching frequency at the polymer level are not yet fully understood and it is intriguing to visualise how such highly branched glucans as E25 and EPS180 might bind to the enzyme and reorient as synthesis proceeds.

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## Figure captions

Fig. 1. 600 MHz $^{1}$H NMR spectra of a) E25 at $320^{0}$K; b) E25 at $363^{0}$K; c) B1355S at $320^{0}$K; d) B1355S at $363^{0}$K. All spectra were recorded in $D_2O$ with presaturation of the water signal. At $363^{0}$K the HDO chemical shift is very close to the **B**5 signal and presaturation reduces the

intensity of **B**5. At $320^0$K with a reduced presaturation power level none of the polysaccharide signals are perturbed. Signal labelling is as described in the text (*3.3.1*).

Fig. 2. 150 MHz $^{13}$C NMR spectra of a) E25; b) B1355S; c) dextran. All spectra were recorded in $D_2O$ at $363^0$K using the *dept135* sequence. Signal labelling is as described in the text (*3.3.1*).

Fig. 3. Schematic model of a portion of the E25 EPS structure. The labelling scheme is described in *3.3.1*.

**Table captions**

Table 1. Mole percentages of methyl ethers from methylation analyses of extracellular polysaccharides made up of 3- and 6-linked glucose units.

Table 2. $^1$H and $^{13}$C chemical shifts of α-Glc residues in B1355S, E25 and Dextran, measured at $363^0$K. (n.d. not determined; n.a. not applicable). Signal and unit labelling is as described in the text (*3.3.1 & 3.3.6*).

Table 3. (A) Integrals and deconvolution ratios from the $^1$H NMR spectra of E25 and B1355S; (B) Percentages of different types of α-Glc unit in E25 and B1355S. Signal and unit labelling is as described in the text (*3.3.1*).

**Supplementary figure captions**

Fig. S1. *Hsqc-tocsy* spectrum of B1355S at $363^0$K. Negative contour levels are shown in light grey. Traces and signals have been labelled **A**-**F** using the scheme described in the text (*3.3.1 & 3.3.*6) to indicate the parent sugar unit.

Fig. S2. *Hsqc* spectrum of B1355S at $363^0$K (central region only). Cross-peaks have been labelled **A**-**F** using the scheme described in the text (*3.3.1 & 3.3.*6) to indicate the parent sugar unit. The high sensitivity pulse sequence gives rise to some low intensity artefacts (e.g. cross-peaks H-2/ C-3, H-3/ C-2 in addition to the expected H-2/ C-2, H-3/ C-3 etc.). These additional peaks have not been annotated.

Fig. S3. *Hsqc-tocsy* spectrum of E25 at $363^0$K. Negative contour levels are shown in light grey. Traces and signals have been labelled **A**-**F** using the scheme described in the text (*3.3.1 & 3.3.*6) to indicate the parent sugar unit.

Fig. S4. *Hsqc* spectrum of E25 at $363^0$K (central region only). Cross-peaks have been labelled **A**-**F** using the scheme described in the text (*3.3.1 & 3.3.*6) to indicate the parent sugar unit. The high sensitivity pulse sequence gives rise to some low intensity artefacts (e.g. cross-peaks H-2/ C-3, H-3/ C-2 in addition to the expected H-2/ C-2, H-3/ C-3 etc.). These additional peaks have not been annotated.

Fig. S5. *Hsqc* spectrum of dextran at $338^0$K (central region only). Cross-peaks have been labelled **A**-**F** using the scheme in the text (*3.3.1 & 3.3.*6) to indicate the parent sugar unit. The pulse sequence *hsqcetgpprsp.2* was used to minimise the artefact peaks seen in Figs. S2 and S4 since artefact peaks associated with unit **C** would otherwise be comparable in intensity to the genuine cross-peaks from the other units. The artefacts that can still be seen have not been annotated.

Figure 1

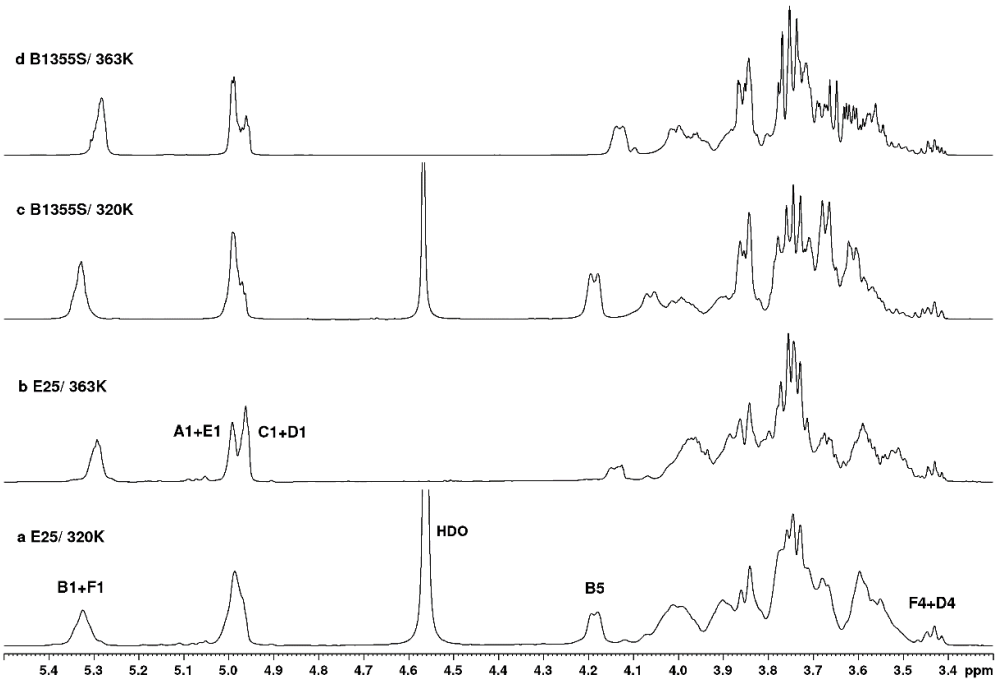

Figure 2

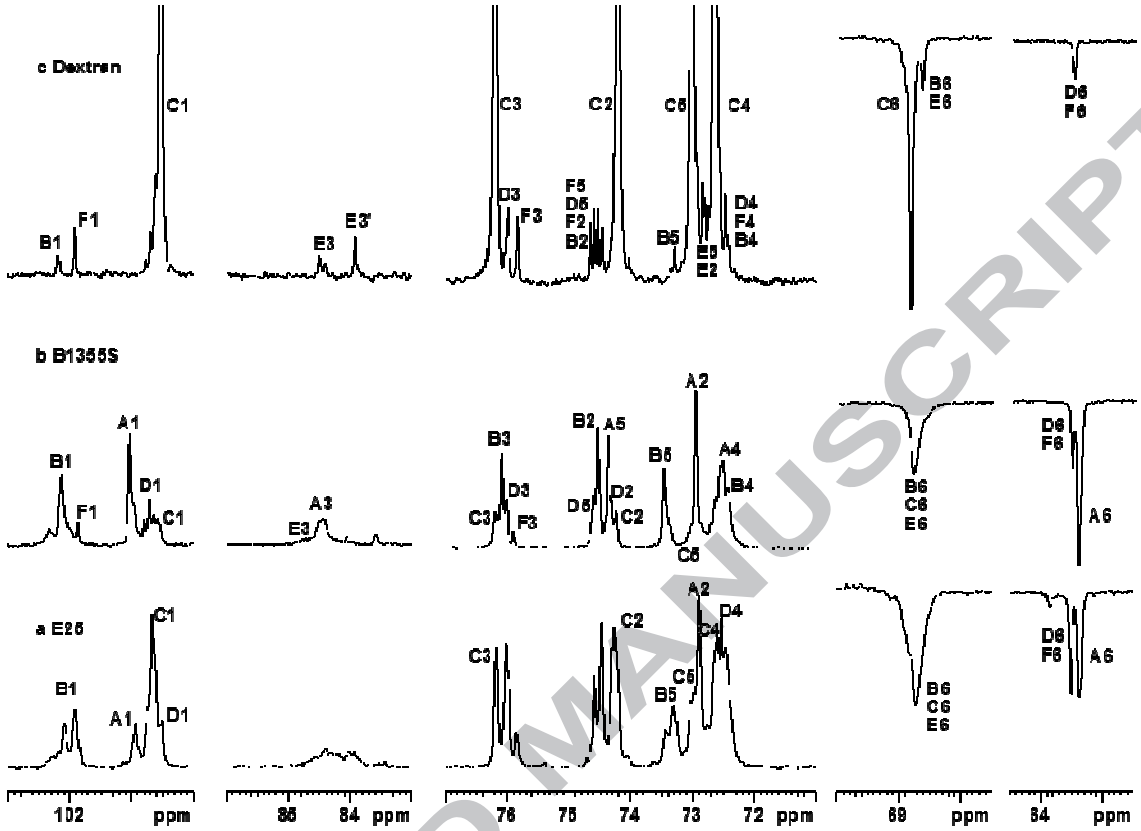

Figure 3

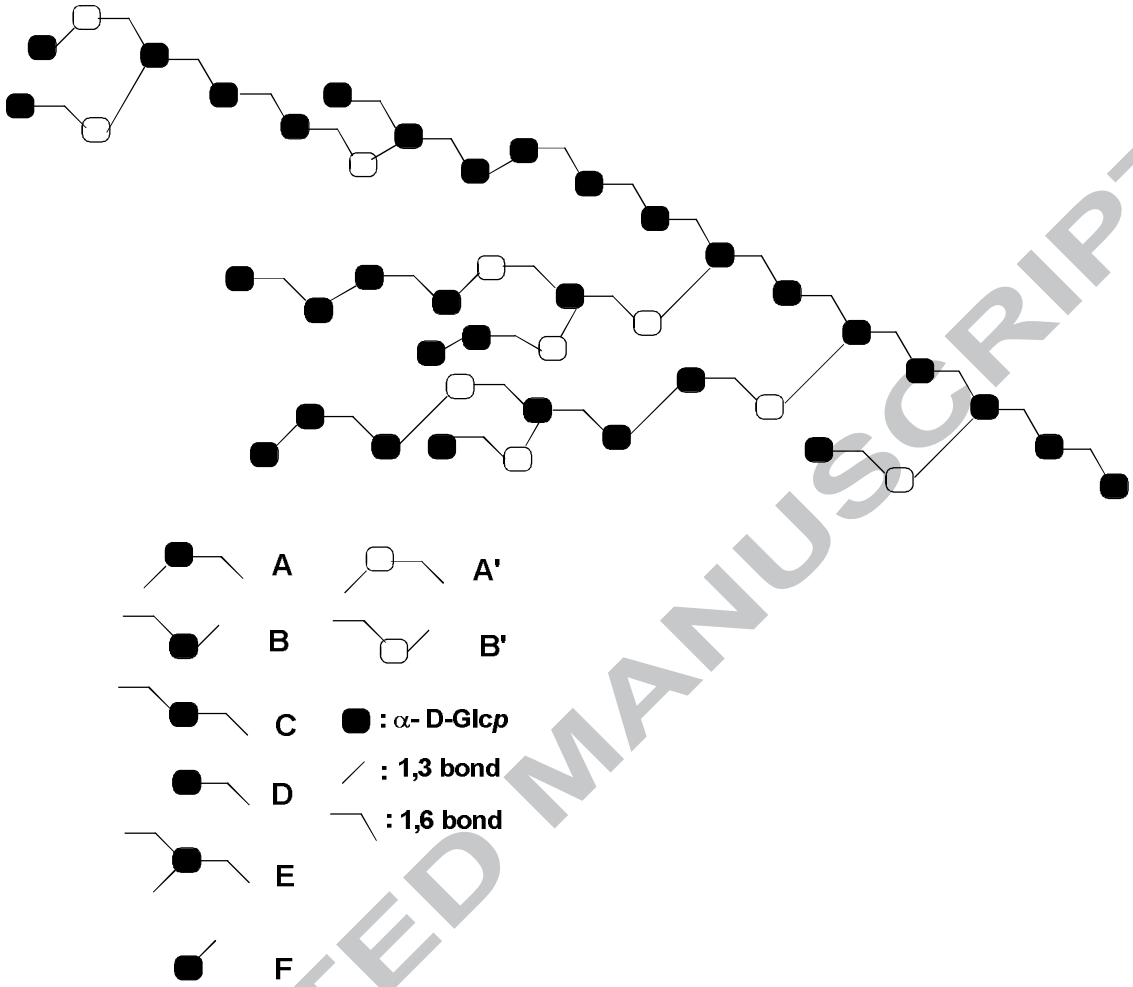

**Table 1.** Mole percentages of methyl ethers from methylation analyses of extracellular polysaccharides made up of 3- and 6-linked glucose units.

| Methyl ether | Residue type | **E25** | **B1355S** (alternan) | **B1501S** (alternan) | **EPS180**[a] |
|---|---|---|---|---|---|
| 2,3,4,6-tetra-OMe Glc | t-Glc | 19.4 ± 1.8 | 13.1 ± 0.8 | 14.6 ± 2.1 | 12 |
| 2,4,6-tri-OMe Glc | (1,3)-Glc | 18.9 ± 1.7 | 35.4 ± 0.3 | 31.5 ± 1.2 | 24 |
| 2,3,4-tri-OMe Glc | (1,6)-Glc | 53.9 ± 1.2 | 40.2 ± 0.1 | 43.5 ± 1.0 | 52 |
| 2,4-di-OMe Glc | (1,3,6)-Glc | 7.8 ± 1.7 | 11.3 ± 0.4 | 10.5 ± 0.2 | 12 |

[a] van Leeuwen et al., 2008

**Table 2.** $^{1}H$ and $^{13}C$ chemical shifts of α-Glc residues in B1355S, E25 and Dextran, measured at $363^{0}K$ (n.d. not determined; n.a. not applicable). Signal labelling is as described in the text (*3.3.1 & 3.3.6*).

| | **A** (1,3)→6 | | **B** (1,6)→3 | | **C** (1,6)→6 | | **D** t→6 | | **E** (1,3,6)→6 | | **F** t→3 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **B1355S** | | | | | | | | | | | | |
| | H | C | H | C | H | C | H | C | H | C | H | C |
| 1 | 4.99 | 101.04, 100.63 | 5.28 | 102.16, 101.99 | 4.96 | 100.60 | 4.96 | 100.71 | 4.99 | 100.96 | 5.30 | 101.89 |
| 2 | 3.68 | 72.96 | 3.62 | 74.55 | 3.58 | 74.25 | 3.55 | 74.31 | 3.65 | 72.68 | 3.58 | 74.58 |
| 3 | 3.85 | 84.47 | 3.75 | 76.10 | 3.73 | 76.20 | 3.73 | 76.03 | 3.86 | 84.72 | 3.75 | 75.91 |
| 4 | 3.65 | 72.56 | 3.56 | 72.44 | 3.50 | 72.65 | 3.43 | 72.61 | 3.72 | 72.57 | 3.44 | 72.55 |
| 5 | 3.72 | 74.38 | 4.13 | 73.46 | 3.89 | 73.06 | 3.71 | 74.62 | 3.89 | 72.86 | 3.98 | 74.65 |
| 6 | 3.76 | 63.44 | 3.72 | 68.76 | 3.79 | 68.81 | 3.76 | 63.55 | 3.81 | 68.81 | 3.75 | 63.55 |
| 6´ | 3.85 | | 4.01 | | 3.96 | | 3.85 | | 3.95 | | 3.84 | |
| **E25** | | | | | | | | | | | | |
| 1 | 5.00 | 100.95, 100.63[a] | 5.31 | 102.10, 101.92[b] | 4.97 | 100.69 | 4.97 | 100.67 | 5.00 | 100.90 | 5.32 | 101.89 |
| 2 | 3.69 | 72.91 | 3.61 | 74.49 | 3.59 | 74.24 | 3.57 | 74.28 | 3.66 | 72.64 | n. d. | n. d. |
| 3 | 3.84 | 84.44 | 3.76 | 76.03 | 3.75 | 76.19 | 3.74 | 75.98 | 3.84 | 84.58 | 3.77 | 75.85 |
| 4 | 3.67 | 72.55 | 3.58 | 72.41 | 3.51 | 72.65 | 3.44 | 72.55 | 3.73 | 72.66 | 3.45 | 72.51 |
| 5 | 3.75 | 74.30 | 4.15 | 73.45, 73.31[c] | 3.89 | 73.02 | 3.72 | 74.59 | 3.93 | 72.77 | 3.98 | 74.65 |
| 6 | 3.77 | 63.38 | 3.75 | 68.68 | 3.80 | 68.75 | 3.77 | 63.52 | 3.80 | 68.72 | 3.77 | 63.52 |
| 6´ | 3.86 | | 4.01 | | 3.95 | | 3.86 | | 3.96 | | 3.86 | |
| **Dextran** | | | | | | | | | | | | |
| 1 | n.a. | n.a. | 5.29 | 102.17 | 4.97 | 100.53 | 4.96 | 100.61 | 4.99, 5.01 | 100.62, 100.53 | 5.30 | 101.92 |
| 2 | n.a. | n.a. | 3.62 | 74.47 | 3.58 | 74.21 | 3.55 | 74.23 | 3.68 | 72.89 | 3.60 | 74.54 |
| 3 | n.a. | n.a. | 3.75 | 76.00 | 3.73 | 76.19 | 3.73 | 76.02 | 3.85, 3.83 | 83.64, 84.25[d] | 3.76 | 75.84 |
| 4 | n.a. | n.a. | 3.54 | 72.42 | 3.50 | 72.63 | 3.43 | 72.54 | 3.74 | 72.66 | 3.45 | 72.48 |
| 5 | n.a. | n.a. | 4.15 | 73.29 | 3.90 | 73.01 | 3.72 | 74.58 | 3.92 | 72.79 | 3.98 | 74.65 |
| 6 | n.a. | n.a. | 3.75 | 68.73 | 3.79 | 68.83 | 3.77 | 63.53 | 3.78 | 68.65 | 3.77 | 63.51 |
| 6´ | n.a. | | 4.01 | | 3.96 | | 3.85 | | 3.97 | | 3.85 | |

[a] **A1**, **A1´** respectively

[b] **B1**, **B1´** respectively

[c] **B5**, **B5´** respectively

[d] **E3´**, **E3** respectively

**Table 3.** (A) Integrals and deconvolution ratios from the $^{1}$H NMR spectra of E25 and B1355S; (B) Percentages of different types of α-Glc unit in E25 and B1355S. Signal and unit labelling is as described in the text (*3.3.1*).

(A)

| **Signals** | **δ ppm** | **E25** | **B1355S** |
|---|---|---|---|
| *Integration* | | *Integral* | *Integral* |
| **B**1+**F**1[a] | 5.30 | 1.0 | 1.0 |
| (**A**1+**E**1)+(**C**1+**D**1)[a] | 4.99 | 2.0 | 1.48 |
| **B**5[a] | 4.20 | 0.85 | 0.92 |
| **D**4+**F**4[a] | 3.44 | 0.45 | 0.31 |
| *Deconvolution* | | *Ratio* | *Ratio* |
| (**A**1+**E**1): (**C**1+**D**1)[b] | 4.99 | 1: 1.09 | 2.26: 1 |
| **F**4: **D**4[c] | 3.44 | 2.61: 1 | 1.71: 1 |

[a] integrals from 320$^{0}$K spectra

[b] by deconvolution of the 363$^{0}$K spectrum

[c] by deconvolution of the 338$^{0}$K and 363$^{0}$K spectra (average)

(B)

| **Code** | **α-Glc Unit** | **E25** | | **B1355S** | |
|---|---|---|---|---|---|
| | | *Integral* | *Percentage %* | *Integral* | *Percentage %* |
| **A** | (1,3)→6 | 0.51 | 17 | 0.72 | 29 |
| **B** | (1,6)→3 | 0.88 | 29 | 0.89 | 36 |
| **C** | (1,6)→6 | 0.71 | 24 | 0.25 | 10 |
| **D** | t→6 | 0.33 | 11 | 0.20 | 8 |
| **E** | (1,3,6)→6 | 0.45 | 15 | 0.31 | 12 |
| **F** | t→3 | 0.12 | 4 | 0.11 | 4 |

- A sourdough isolate *Lactobacillus brevis* E25 produced a highly branched α-glucan with (α1→ 3) and (α1→6) glycosidic linkages.
- A modified medium was used to isolate the E25 exopolysaccharide (EPS) without contamination.
- The structure of E25 EPS was determined and its NMR parameters are reported together with those of alternan and a commercial dextran.
- E25 EPS contains a higher proportion of (1,6)Glc→6 units and is more highly branched than alternan.
- Understanding the production of glucans in food environments as well as at the biochemical level is crucial for future studies.